

Consumer and
Corporate Affairs CanadaConsommateur
et Affaires Corporatives Canada

(11) (A) No. 1 271 708

(45) ISSUED 900717

(52) CLASS 167-130

(51) INT. CL. A61K 39/205

(10) (CA) **CANADIAN PATENT** (12)(54) Process for the Preparation of a Rabies Vaccine and
the Vaccine Obtained by This Process(72) Glück, Reinhard;
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Switzerland(73) Granted to Schweizerisches Serum-Und Impfinstitut
Und Institut Zur Erforschung Der
Infektionskrankheiten, Switzerland

(21) APPLICATION No. 517,612

(22) FILED 860909

(30) PRIORITY DATE (CH) Switzerland (04 999/83-9)
851122

No. OF CLAIMS 38 - NO DRAWING

Canada

ASTA BUREAU DE METAIERS OFFICIELS, OTTAWA
CANADIAN

ABSTRACT OF THE DISCLOSURE

The invention relates to a process for the preparation of a rabies vaccine by multiplication of rabies viruses in animal nerve tissue or poultry embryos, harvesting of the
5 viruses from the nerve tissue or from the heads of the embryos, enriching the viral preparation, inactivating thereof, and preparing a vaccine. In one aspect, the process comprises homogenizing the nerve tissue or embryo heads and harvesting of the viruses therefrom by avoiding the use of a
10 mixer, and thus preventing damage to and fragmentation of the viruses. The nerve tissue, the embryo heads or their contents are comminuted in a manner which preserves cell and viral integrity. The preparation is then treated by separating the complete live viruses which are capable of
15 multiplication from the resulting cell suspension, delipidating by extraction with a water-immiscible organic solvent and then further selectively concentrating the viruses thereof.

The invention also relates to a myelin-free rabies
20 vaccine which has been obtained by the process described above from animal nerve tissue or poultry embryo head tissue containing rabies viruses.

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BACKGROUND OF THE INVENTIONField of the Invention

The present invention relates to a new, economic process for obtaining a rabies vaccine comprising obtaining whole
5 live viruses and rendering the viruses thereof incapable of replicating by chemical treatment. This invention also relates to a vaccine obtained by this process, which is by reason of its high purity, distinguished by a high specific activity and the absence of undesirable secondary reactions
10 when inoculated to human subjects.

Description of the Background

Most rabies vaccines have up until the present time been obtained by multiplication of the rabies virus in living animals such as mice, rats, rabbits, sheep, etc. However,
15 the thus obtained virus-containing preparations contain considerable amounts of myelin and elicit detrimental side effects.

In recent times, rabies vaccines have also been obtained from viruses multiplied in poultry embryos. This method has
20 the advantage, in principle, that the thus obtained virus-containing tissue contains hardly any injurious myelin. After multiplication of the viruses in poultry embryos, these embryos are completely homogenized in toto in a mixer or blender. In this manner, however, it is only possible to
25 incompletely separate from this pasty homogenate the virus

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constituents from heterologous protein which may initiate
undesired secondary reactions upon inoculation. This is also
the case with vaccines obtained from brains of living
animals which have been infected with rabies. On repeated
5 inoculation - indispensable in the case of hunters, forestry
workers, veterinarians, etc. - these secondary reactions may
increase considerably and result in violent allergic defense
reactions against the heterologous proteins.

The quality of embryo vaccines has been somewhat
10 improved by using only the heads of the embryos to obtain the
vaccines. Since, in comparison, embryo heads carry an
essentially higher concentration of the viruses, the vaccines
prepared only from embryo heads have a correspondingly lower
content of byproducts and cause fewer side effects (German
15 Patent 3,009,064; U.S. Patent 4,255,520).

However, in the course of preparing vaccines from nerve
tissue of animals (from embryos or from embryo heads),
viruses are often damaged or fragmented when the virus-
containing tissues are homogenized with a mixer or blender.
20 This considerably reduces the activity of the vaccine
prepared from homogenates of this type and makes its purifi-
cation more difficult since large amounts of proteins
and liquids are released from the fragmented cells.

A slightly better vaccine has only been obtained by
25 multiplication of the rabies viruses by in vitro culturing
human diploid cells (HDC) (H. Koprowski, "Vaccine for man

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- prepared in human diploid cells", Laboratory Techniques in Rabies by M.M. Kaplan and H. Koprowski, WHO Monograph Series No. 23, Chapter 28, pp. 256-60 (1973); T.J. Wiktor, Develop. Biol. Standard, Vol. 37, pp. 256-66, S. Karger, Basel 1978, "Production and control of rabies vaccines made on diploid cells"; T.J. Wiktor et al. "Development and clinical trial of rabies vaccine of tissue culture origin", Develop. Biol. Standard, Vol. 40, pp. 3-9 (1978)). The thus obtained vaccines contain human proteins as contaminants.
- 10 Such proteins, however, although producing fewer secondary reactions than do heterologous proteins, still produce some.

A considerable disadvantage of this method is the relatively low multiplication rate of the rabies viruses in diploid fibroblast cells. This requires the use of a 10- to 25-fold greater concentration of the vaccine. Hence, this method is not efficient enough to meet world-wide demand for rabies vaccine in an economically feasible manner.

- The preparation of a rabies vaccine in duck embryo cell cultures is described in U.S. Patent 3,674,862. In this process, however, the multiplication rate in cell cultures is limited, (U.S. Patent 3,973,000 describes a method for the enrichment of rabies viruses by density gradient centrifugation; M. Rolle and A. Mayr; Mikrobiologie, Infektions- und Seuchenlehre, Stuttgart (Microbiology, Infection and epidemiology):489-493 Stuttgart (1978) describe the traditional preparation of duck embryo rabies vaccine).

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Thus, there is a pressing need for a new and highly active rabies vaccine which contains mechanically intact viruses with fully retained antigenic activity, which is straightforward to prepare and thus not too costly, and
5 free of side effects. Such vaccine would be an effective and well-tolerated vaccine which has long been sought for world-wide control of the fearsome and fatal rabies disease.

SUMMARY OF THE INVENTION

The present invention provides a process for obtaining
10 inactivated rabies viruses which are substantially myelin-free, comprising:

- (1) intracerebrally inoculating an experimental animal with whole live rabies viruses;
- (2) allowing for said viruses to multiply;
- 15 (3) comminuting nerve tissue from the animal's brain to obtain a cell suspension, said comminution being conducted in the absence of a mixer to preserve the integrity of the viruses;
- (4) separating live whole viruses from the cell
20 suspension;
- (5) delipidating the live whole viruses; and
- (6) selectively concentrating the viruses; wherein steps (1) through (4) are conducted at least once and up to
3 times.

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In addition, this invention also provides a process for obtaining inactivated rabies viruses which are substantially myelin-free comprising:

- 5 (1) inoculating a poultry embryo egg with whole live rabies viruses;
- (2) allowing for said viruses to multiply;
- (3) comminuting the embryo from the poultry egg to obtain a cell suspension; said comminution being conducted in the absence of a mixer to preserve the integrity of the
- 10 viruses;
- (4) separating live whole viruses from the cell suspension;
- (5) delipidating the live whole viruses; and
- (6) selectively concentrating the attenuated viruses;
- 15 wherein steps (1) through (4) are performed at least once and up to 3 times.

This invention also provides a rabies vaccine comprising inactivated rabies viruses which are substantially myelin-free, said viruses being present in an amount

20 effective to elicit an immunizing response when administered to a subject. The present vaccine may be obtained by the hereinabove processes.

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DESCRIPTION OF THE INVENTION

The present invention relates to an economic process for preparing a rabies vaccina which is of the highest quality when compared to vaccines obtained from viruses multiplied in
5 diploid human cell cultures.

In one aspect of this invention the process comprises

- (1) isolating the rabies viruses which have multiplied in animal nerve tissue or poultry embryos avoiding mechanical damage to or fragmentation of the viruses thereof;
- 10 (2) removing lipids from the resulting viruses by extraction with a water-immiscible organic solvent such as volatile paraffin hydrocarbons or halogenated hydrocarbons such as fluorinated hydrocarbons;
- (3) enriching the delipidated viruses by density
15 gradient centrifugation;
- (4) precipitating the viruses by addition of a polyethylene glycol (e.g., PEG 6000) and concentrating by centrifugation and purifying the live whole viruses.

Step (1) entails extracting the rabies virus by cautious
20 comminution [preserve cell and viral integrity] of the nerve tissue or poultry embryo heads (e.g., duck, chicken or quail), and washing the tissue fragments with a buffer, e.g., phosphate-containing buffer. This step is superior than homogenizing in a mixer or blender since foreign proteins and
25 lipids are solubilized to a lesser extent, the occurrence of

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oxidation products of antigens, proteins and lipids is avoided, and the content of intracellular, incomplete and non-immunizing rabies antigen is diminished. The virus-containing suspension obtained by washing the tissue fragments with an aqueous buffer solution is then removed by differential centrifugation.

At least 95% of the residual protein is discarded by operations 3 and/or 4.

The viruses are then finally inactivated in a known manner, for example by addition of β -propiolactone or tri-(n-butyl)phosphate.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The individual process steps of the inventive process are performed in such a manner that a surprisingly good overall result is achieved. Harvesting viruses only from the heads of the embryos entails producing a high basic concentration of the viruses. The mild treatment of the virus-containing tissue material gives a fine paste, especially upon avoiding the homogenization thereof with a mixer or blender, provides a viral suspension which exhibits substantially no mechanically damaged or fragmented viruses with incomplete antigen content, and which, moreover, contains far fewer foreign materials such as cell debris, proteins and lipids. The remaining lipids can be removed from this viral suspension by extraction, and the proteins

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can be far more completely removed by selective concentration and/or precipitation of the virus than from a pasty homogenate. The vaccine obtained by the process comprising the sequence of steps described is improved by around 90-fold compared with the conventional duck embryo rabies vaccine.

When the preparation of rabies vaccine is obtained by passaging on nerve tissues of animals such as mice, rats, rabbits and sheep, the viruses are multiplied in the living animal by intracerebral inoculation of rabies viruses of standardized seed strains. It must, however, be noted that the multiplication of the viruses in living animals has the disadvantage, compared with the multiplication of the viruses in poultry embryos, that the nerve tissues of living animals contain myelin. This protein is known to give rise to secondary reactions when the vaccine is used, including encephalitis.

After slaughtering the animals, which were previously inoculated with live whole rabies viruses, their brains are removed, comminuted in a manner which preserves the wholeness of the cells and the viruses, and a vaccine is prepared from the resulting cell suspension by the process described hereinabove. Owing to the avoidance of cell fragmentation during the comminution of the virus-containing nerve tissues, less myelin is released than during comminution with a mixer. During the extraction of the lipids with an organic solvent in a later step of the process a further part of the

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still present myelin is removed in such a manner that the vaccine which is finally obtained causes only minimal, if any, secondary reactions, and those which are caused are still highly tolerable.

- 5 The steps of the process must be conducted avoiding the use of a mixer or blender for the homogenization of nerve tissues or of embryos or embryo heads on harvesting of the viruses. This prevents damage to and fragmentation of the viruses by comminution of the nerve tissues or of the embryos
10 or embryo heads and their contents in a manner which preserves the wholeness of the cells and the viruses, separating the complete live viruses which are capable of multiplication from the resulting cell suspension, and purifying the resulting viral suspension, delipidating by
15 extraction with a water-immiscible organic solvent and then selectively concentrating the viral preparation.

- The comminution of the nerve tissue, the embryos or embryo heads or their contents, is carried out with the aid of a meat mincer on a coarse setting, by cutting up or by
20 opening of the heads and comminuting the removed brain tissue in a manner which preserves the integrity of the cells, and therefore the viruses.

- Washing or extracting the viruses from the comminuted tissue is carried out with a buffer solution, preferably with
25 an aqueous phosphate buffer of about pH 7-8, as is known in the art. The removal of the lipids is carried out by

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extraction with a water-immiscible solvent, such as liquid, volatile, optionally halogenated hydrocarbons. Suitable solvents are petroleum ethers such as heptanes, fluorinated and chlorinated ethanes and homologs thereof. However other solvents can also be used. The further concentration of the delipidated viral suspension can be carried out by density gradient centrifugation and/or precipitation with a polyethylene glycol, preferably with PEG 6000, as is known in the art. Suitable types of embryo poultry eggs for the multiplication of rabies viruses are in particular those from ducks, chickens and quails. In general, incubated duck eggs are preferred as the tissue for the multiplication of the viruses. The myelin-free rabies vaccine provided herein may be obtained from poultry embryo head tissue which contains rabies viruses by the process which is described above, which process fully preserves viral integrity.

The processes according to the invention results in a rabies vaccine which, compared with the vaccines obtained by processes hitherto known, exhibits a far better ratio of antigen content to protein content, contain substantially no foreign lipids, and approach in quality an ideal HDC vaccine.

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DETAILED DESCRIPTION OF THE PROCESS

Now the process will be described in relation to each separate step.

Step 1:

- 5 A rabies virus strain which is suitable for the preparation of the vaccine is adapted to the intended viral host by appropriate passages on the embryonal cells of poultry eggs or in mice, rats, rabbits or sheep, among others.
- 10 Attenuated rabies viruses are, for example, inoculated into the yolk sac of fertilized poultry eggs which have undergone initial incubation and in which an embryo has started to develop. After about two weeks, the embryos are removed and their heads are harvested. The embryo heads are
- 15 comminuted in a manner which preserves cell and viral integrity in a meat mincer.
- Alternatively, the head of the embryo is cut open and the brain tissue is removed and comminuted. The multiplication of the viruses may also be undertaken in
- 20 living animals. In such case, animals which are only a few days old (mice, rats, rabbits, lambs, etc.) are usually inoculated intracerebrally with the same species-specific attenuated seed virus.

- After about 10-30 days the animals are sacrificed,
- 25 and the brains are removed by operation and comminuted in a

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manner which preserves cell and viral integrity. The extraction of the rabies virus from the comminuted tissue is carried out by washing the tissue fragments with a phosphate-containing buffer. A suitable phosphate buffer is one comprising, e.g., 0.75% by weight of disodium hydrogen phosphate (Na_2HPO_4), 0.145% by weight of potassium dihydrogen phosphate (KH_2PO_4) and 0.48% by weight of sodium chloride in distilled water, pH 7.4. However, other buffer solutions known in the art may also be used. It is equally possible to use for the extraction, stabilizers and salt solutions which are customarily used for the preparation of viral vaccine suspensions, or even deionized water as long as the pH is in the range between 7 and 8.

The suspension containing the viral antigens is separated from the tissue by differential centrifugation at about 10,000-15,000 x g (g being the acceleration of gravity). The remaining tissue sediment can be used for further extractions, by which means a yield of about 30% of viral antigen is possible. The two or more virus-containing extracts are combined and then filtered.

Step 2:

The foreign lipids still remaining in the viral suspension are removed by extraction with a water-immiscible organic solvent, such as, e.g., with an hydrocarbon, optionally halogenated and preferably fluorinated.

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Subsequently, the antigen extract (the viral suspension) is enriched by density gradient centrifugation in a manner known per se at 15,000-90,000 x g using a buffer and sugar solutions of various concentrations, by increasing the sugar concentration in the buffer in a manner known in the art. Alternatively, the viral suspension can be concentrated by precipitation with polyethylene glycol.

Step 3:

The density gradient centrifugation is carried out in a manner known per se at 15,000 to 19,000 x g using sugar solutions of various concentrations and buffer solutions, by increasing the sugar concentration in a buffer solution. For this purpose, the prepurified suspension is pumped at 15,000 to 90,000 x g at a flow rate of, e.g., 4 litres/h over a step gradient of an increasing concentration of sugar (usually, sucrose from 15 to 55 %) which has previously been introduced. The fractions collected from the various densities are then subjected to tests for density, the contents of lipids, nucleoproteins and glycoproteins, and sterility.

The antigen-containing fractions are pooled, tested once more, and then processed further to obtain the vaccine. Physiological saline solutions of any type, e.g., the phosphate buffer mentioned above, can be used for dilution in a manner known per se (Duck embryo rabies vaccine:

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J.M. Hopkins, Laboratory Techniques in Rabies by M.M. Kaplan et al., WHO Geneva 1973, Chapter 27, pages 243-55; Density gradient centrifugation: J. Hilfenhaus et al., J. Biol. Standard. 4:263-271 (1973); M. Majer et al., Develop. Biol. Standard. 37:267-271 (1977); and P. Atanasiu et al., Develop. Biol. Standard. 40:35-44.

Step 4:

In addition or alternative to the enrichment of the virus concentration by density gradient centrifugation, the prepurified, and usually enriched, viral suspension can be further concentrated and purified by precipitation with a polyethylene glycol, preferably free of heterologous protein. For this purpose, the pH of the viral suspension can be adjusted to about 8. After addition of a polyethylene glycol (e.g., PEG 6000) to a final concentration of 6% by weight, the suspension is stirred for at least one hour and the virus is precipitated by subsequent centrifugation at 10,000--15,000 x g. The viral sediment is then resuspended in a stabilizer composed of a solution containing lactose and physiological gelatin (E.M. Mikhailovsky et al., Ann. Inst. Pasteur 121:563-568 (1971); James McGarry et al., Virology 40:745-746 (1970).

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Step 5:

The intact live viruses capable of multiplication which are present in the resulting viral concentrate are now inactivated. Beta-propiolactone (BPL) is usually used for the inactivation (G.A. LeGrippe, Annals New York Acad. of Sci. 83:578-94 (1960). However other substances are also suitable for this purpose such as tri(n-butyl) phosphate (H. Tint et al., Symposia series in "A new tissue culture rabies vaccine, inactivated and disaggregated with tri-(n-butyl) phosphate" Immunobiol. Standard. (Karger, Basel) 21:132-144; T.J. Wiktor et al., Develop. Biol. Standard. 40:3-9 (1978).

The vaccine concentrate obtained by the new process differs from commercially available rabies vaccines in its high content of antigen value units per mg of nitrogen [measured using the standard NIH test in mice and the antibody binding test in the RFFIT]. Preferably, the vaccine contains more than 10 antigen value units per mg of nitrogen, and still more preferably more than 15 units per mg of nitrogen, but always more than 8 units per mg of nitrogen. As a rule, the same can be obtained using unborn embryos which do not as yet feel pain and in which the brain tissue, which is just in the process of development, appears to be still free of myelin (M. Abdussalen et al., "The problem of anti-rabies vaccination", International conference on the application of

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vaccine against assay viral rickettsial and bacterial diseases of man, Pan. Am. Health Org. (PAHO), Sc. pub. No. 226:54-59 (1970); and P. Fenle, "The status of existing rabies vaccines", *ibid.* pages 60-65).

5 Step 6:

The vaccine resulting after the inactivation can be dispensed into vials and can then be freeze-dried. It may be reconstituted for use by dissolution or suspension using distilled water.

- 10 As is well understood by those skilled in the art of viral purification, additional steps may be included to further purify the rabies virus.

It is possible by the process which has been described herein to prepare unlimited, or at least adequate, amounts of
15 a valuable and innocuous rabies vaccine in an economic and relatively straightforward manner. The preparation of such quality rabies vaccine by multiplication of the viruses in human diploid cell cultures (HDC) is highly impossible as a consequence of the low efficiency of the substrate.

- 20 It is noteworthy that by an order of February 1979, the CDC has restricted the use of human diploid cell rabies vaccine to people having developed life-threatening side effects after administration of the duck embryo vaccine or who were incapable of acquiring an appropriate titer of
25 antibodies. The reason given for this is inadequate

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productivity of the human diploid cell cultures (See also, Morbidity and Mortality Weekly Report (MMWR) 27:333, 413 (1978)).

The rabies vaccine prepared by the process according to
5 the invention is at least equivalent to an HDC vaccine in
which the viruses have been multiplied in human diploid cell
cultures (See, Example 1 hereinbelow). No side effects have
been observed upon administration of this vaccine up to the
present time, thereby making available for medical use a
10 rabies vaccine of excellent value and effectiveness and which
has negligible side effects.

When the antigen of this invention is used to induce
immune response in a human or animal, it is administered in
an amount sufficient to elicit an immunizing response. The
15 amount of antigen may be adjusted by a clinician doing the
administration, as commonly occurs in the administration of
vaccines and other viral agents which induce immunizing
responses. Suitable vaccine unit amounts are between about
2.5 units and 10 units, preferably between about 4
20 units and 6 units. Although a single administration
induces an immune response, multiple administrations may be
carried out if desired or if so required in accordance with
schedules known per se. The route of administration can be
any of the routes generally used for rabies vaccines, such as
25 by injection subcutaneously, intramuscularly and the
like.

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Having now generally described this invention, the same will be better understood by reference to certain specific examples, which are included herein for purposes of illustration only and are not intended to be limiting of the invention or any embodiment thereof, unless so specified.

EXAMPLES

Example 1 - PREPARATION OF A PURIFIED DUCK EMBRYO RABIES VACCINE

1. Preparation of the virus suspension

- (a) The "Wistar rabies, PM (Pitman-Moore) 8RDCS" virus strain from the Wistar Institute, Philadelphia, or another rabies virus strain suitable for the preparation of a vaccine was adapted to the embryo cells before actual use by intracerebral passage in mice and repeated passage by inoculation in duck eggs which have undergone initial incubation. The viruses used for the preparation of the vaccine are those from a passage with a particularly high titer and which have already proved to be suitable in the preparation of rabies vaccine in accordance with the method of J.M. Hosking, "Laboratory Techniques", in Rabies by Kaplan et al., WHO, 27:243-55 Duck Embryo Vaccine (1973).

Fertilized duck eggs from healthy stocks were incubated at a temperature of $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and a humidity of 65-70%. After six days they are candled with UV light and

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unsuitable eggs are rejected. On day 7 of incubation, the rabies virus was inoculated directly into the yolk sac of the eggs in which an embryo was developing. The incubation was continued and 10-14 days later the eggs were again candled with UV light. The eggs in which the embryos continued to develop well were opened under sterile conditions, and the embryos were removed and decapitated. The heads were stored individually under sterile conditions in the vapor phase over liquid nitrogen until the sterility tests were complete. Groups of 40-60 of the sterile heads were combined into a pool with the addition of a defined amount of a stabilizer. The sterility of each pool was again tested. In addition to the stabilizer, it was also possible to use a NaCl/phosphate buffer comprising 0.75% disodium hydrogen phosphate, 0.145% potassium dihydrogen phosphate and 0.48% sodium chloride in distilled water, or other saline solutions as are customary for the purpose of diluting vaccines, even desalinated water, as long as the pH was in the range between 7 and 8.

The rabies virus extract was obtained by comminution of the above mentioned sterile embryo heads using a meat mincer. The tissue fragments were washed twice with a phosphate-containing buffer. After centrifugation at 10,000-15,000 x g and at a temperature of 2-8°C the infectious virus was collected in the supernatant fraction. Remaining brain particles or other lipid-containing tissues

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were removed by subsequent filtration through a gauze filter system. The remaining residues of head tissue can be extracted once more and filtered by use of the same process, by which means a higher antigen yield of about 10% is achieved. The sediment was again suspended in a phosphate-containing buffer and stirred for at least one hour at low temperature (1-4°C) before the centrifugation and filtration.

(b) A subsequent, virtually complete delipidation was carried out by mixing the resulting viral suspension with an inert liquid hydrocarbon solvent with a relatively low density, such as, for example, n-heptane. Homogenization was carried out in every case under a glass ball containing nitrogen gas. The viral suspension was pumped through a mixer system, e.g., Virtis mixer, at a constant flow rate of, e.g., 500 ml/min. At the same time, the n-heptane was pumped into the mixer system at a rate of 50 ml/min. The lipid-containing phase was removed by centrifugation at 10,000-15,000 x g. Traces of the dissolved hydrocarbon solvent were then removed from the delipidated virus extract by allowing an inert gas such as, e.g., nitrogen, to bubble through the aqueous phase and maintaining the aqueous phase under vacuum at 4°C for a period of about 15 hours.

(c) An alternative process for the hydrocarbon delipidation is as follows. Sterile embryo heads may be

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comminuted, extracted and filtered as described under (1a).
The removal of the foreign lipids may then be carried out by
using the fluorinated hydrocarbon solvent
1,1,2-trichlorotrifluoroethane. The individual working
5 steps remain the same.

11. Concentration and further purification of the virus
suspension

(a) The prepurified rabies viral suspension prepared by
the process described above had a viral titer between 10^7 and
10 10^8 MLD₅₀/ml. This material was further purified and
concentrated by centrifugating once or twice on a linear sucrose
gradient (15-55%) at 75,000-90,000 x g. A concentration
factor of 100:1 was attained in this manner. The
glycoprotein and nucleoprotein content (before and after
15 solubilization of the virus membrane with Triton X 100,
that is to say election of the intact virions), the virus
titer, the density and the sterility of the gradient
fractions were tested. Sterile fractions with a ratio of
rabies glycoprotein to nucleoprotein which corresponds to
20 that of the purified whole virion solution, and with a very
high infectious titer (for example 10^9 - 10^{10} MLD₅₀/ml) were
combined and reserved for further processing.

(b) A further purification and concentration of the
viral suspension can be achieved by polyethylene glycol (PEG)
25 precipitation. For this purpose, PEG 6000 (Siegfried A.G.,

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Zofingen, Switzerland) was dissolved in a 30% strength phosphate-containing buffer solution (pH 8.0). This stock PEG solution was sterilized in an autoclave and stored at 4°C. The viral suspension which was adjusted to a pH of 8.0 with a 10% NaOH solution was then precipitated with the stock PEG solution at a final dilution of 5%. The mixture was stirred at a temperature of 4°C for at least one hour. The rabies virus can then be sedimented by centrifugation at a speed of 10,000-15,000 x g over a period of 30 min. The removed virus was again suspended with a stabilizer to the final volume and was reserved for further processing.

III. Formulation of the viral concentrates

Pretested viral concentrates were combined and diluted with a suitable stabilizer, for example sodium phosphate buffer (pH 7.4), with a physiological sodium chloride solution, or with another stabilizer which has already been described (see, Hoskins, l.c.) to a concentration of about $10^{7.5}$ MLD₅₀/ml. Sterility and virus titer were tested again.

20 IV. Inactivation of the viruses

For the inactivation with beta-propiolactone, the final volume of the viral suspension was maintained at a temperature of 1-4°C with continuous stirring. Freshly prepared, ice-cold aqueous beta-propiolactone solution was

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added in an amount such that a concentration of 1:4,000 was attained. After the suspension was stirred at a temperature of 4°C for 5 min, it was transferred into a second vessel and stirred for a further 40 hours; the pH and temperature were continuously monitored. A decrease in the pH was taken as a measure of BPL hydrolysis. As recorded, the pH fell from about 8.0 to about 7.4. At the end of the inactivation, thiomersal (o-(ethylmercurio)-benzoic acid) was added until the concentration of this antiseptic substance was 1:10,000.

V. Freeze-drying

The inactivated viral suspension obtained in accordance with section IV was dispensed in single doses of 1 ml into 3 ml vials, freeze-drying stoppers were placed loosely on top, and the vaccine was freeze-dried in vacuo. When the drying process was completed the stoppers were pushed in tight and the vials were closed with metal caps to assure the tightness of the vials. The vials were then stored at a temperature of -20°C.

VI. Reconstitution to give the vaccine ready for use, and use of this vaccine

Prior to its use, 1 ml of sterile distilled water was injected through the rubber stopper into each vial. The vial was then shaken cautiously, without forming a foam, until the

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vaccine was completely dissolved. The entire content of the vial was then injected subcutaneously into the upper arm of the subject.

VII. Quality control of the final product - tests

5 The quality control procedures comprised: the determination of the antigenicity, sterility, inactivity, innocuousness and contents of nitrogen, cholesterol, NaCl, BPL residues and thiomersal.

Antigenicity:

10 Antigens were tested in accordance with standard instructions of the National Institute of Health, USA. Their ability to bind antibodies in the RFFIT test was also measured (R.J. Arko et al., Laboratory Techniques in Rabies, 3rd edition, WHO Monograph Series 23:265-267 (1973); and
15 J.S. Smith et al., Lab. techn. in Rab., 3rd edition, WHO Monograph Series 23:354 to 357 (1973).

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Sterility:

All the final products for use were proven to be sterile.

Inactivity:

- 5 This was tested in every case on three young rabbits and ten mice, which, after intracerebral inoculation of the reconstituted vaccine, were observed for 14 days. The animals showed no signs of disease in any case.

10 Innocuousness:

- Three guinea pigs received 5 ml intraperitoneal doses of the reconstituted vaccine solution, and 3 mice received 0.5 ml i.v. doses. In no case did the animals show reactions differing from normal.

- 15 The stability of the vaccine obtained in accordance with the above description in the freeze-dried form was also tested. Efficacy (AGV-U/ml as a percentage of the initial figure (0)) was preserved after storage at the stated temperature for 3 months.

- 20 (a) Stability of the vaccine obtained in accordance with Example 1 (concentration in accordance with 2a) in the freeze-dried form. The

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activity (AGV-U/ml) as a percentage of the initial figure (0 figure) is shown in Table 1, hereinbelow.

Table 1: Activity of the Vaccine (Example 1-2a)

Batch number	0 figure AGV-U/ml	+ 37°C 1 month	+ 37°C 2 months
83 Ly III T16	6.7	100%	110%
83 Ly III T18	7.3	93%	92%

(b) Stability of the rabies vaccine obtained in accordance with Example 1 (concentration in accordance with 2b) in the freeze-dried form. The activity (AGV-U/ml) as a percentage of the initial figure (0 figure) is shown in Table 2 hereinbelow.

Table 2: Activity of the Vaccine (Example 1-2b)

Batch number	0 figure AGV-U/ml	+ 37°C 1 month	+ 37°C 2 months
83 Ly III T15	5.0	148%	98%
83 Ly III T19	8.2	107%	104%
83 Ly III T20	9.7	144%	76%
83 Ly III T21	15.3	124%	92%
83 Ly III T22	8.5	165%	105%
83 Ly III T23	13.4	105%	112%
83 Ly III T23	9.5	147%	126%

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(c) The activity of the rabies vaccine obtained in accordance with Example 1 in a dog after s.c. inoculation is shown in Table 3 hereinbelow..

Table 3: Activity of Vaccine (Example 1, dog)

Number of inoculated animals with more than	Vaccine according to (sa), 7 dogs	Vaccine according to (2b), 8 dogs
0.5 IU	100%	100%
1	85%	88%
2	43%	75%

IU = international units of antibody content

(d) Comparative activity in humans of the rabies vaccine obtained in accordance with Example 1 and the HCD vaccine (Behring).

The activity of the new vaccine was compared with that of the HCD vaccine (Behring). Table 4 hereinbelow shows the percentage of subjects which immunologically reacted by forming antibodies after administration of one of these vaccines, in general 0.5 IU being regarded as conferring protection (inoculation on days 0, 3, 7, 14 and 28).

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Table 4: Comparative Activity of Inventive Vaccine and HDC Vaccine

Antibody titer (RFFIT) (day 14)	Vaccine (Ex. 1 (2b))	Vaccine (Ex. (2a))	HDC vaccine (Bering-Werke)
	3 consecu- tive batches 54 subjects	2 consecu- tive batches 15 subjects	AGV: 6.3 IU 20 subjects
0.5 IU	100%	100%	100%
2	98%	100%	100%
5	80%	80%	80%
10	45%	50%	50%
15	28%	47%	30%

IU = international units of antibody content

Results

The rabies vaccine prepared by the process according to the invention proved in the clinical trial to be of at least equal quality to an HDC vaccine, wherein the viruses had been multiplied in human diploid cell cultures (HDC).

Example 2 - Preparation Of A Purified Duck Embryo Vaccine.

Rabies viruses were multiplied in duck eggs which have undergone initial incubation as described in Example 1. The viruses were separated from the embryo heads by cutting the heads open, removing the brain tissue and comminuting in a

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manner preserving cell and viral integrity, and were harvested by resuspension in a phosphate buffer solution.

The viral suspension was further processed to give a vaccine as in Example 1.

5 Example 3 - Purified Duck Embryo Vaccine.

A highly concentrated viral suspension was prepared in accordance with Example 1 and was inactivated by treatment with tri-(n-butyl) phosphate. After inactivation, the concentrate was freeze-dried.

10 Example 4 - Preparation Of Purified Chicken Embryo Vaccine.

Chicken eggs are incubated at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ under a humidity of 60-75% for 7 days. On day 7 of the incubation, the inoculum virus was directly inoculated into the yolk sac of the embryos undergoing development in the eggs. The incubation continued. Seven days later, the eggs were opened and the embryos were removed and dismembered. The heads, the spinal cord and the trunks were processed separately. They were comminuted in a manner such that they provided a 10% strength tissue suspension (that is to say a suspension of 10% by weight of embryo tissue). The viral concentration was titrated using Antibody binding test in the RFFIT (rapid Fluorescent focus Inhibition Test). Three series of tests were carried out; the results of which are shown in the table hereinbelow.

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Table 5: Preparation of Chicken Embryo Vaccine

	Viral concentration (ID 50/ml)
Head	7.25 / 7.4 & 7.8
Spinal cord	6.55 & 7.8
Trunk	6.0 / 6.6 & 6.8

ID = 50/ml = virus titer \times log 10 for the minimum concentration for infection of 50% of the tissue cultures.

It was found that the tissue of the central nervous system (CNS) contained about 10 times as much viruses as the trunk without the CNS.

On the basis of these results, the preparation of the chicken embryo vaccine by the method described in Example 1 was carried out only with the embryo heads. In the present case, the rabies viruses which had been adapted to chicken cells was multiplied in partially incubated chicken eggs, e.g., by the method of H. Koprowsky (Laboratory technique in Rabies by M.M. Kaplan et al., WHO Geneva, Chapter 26, pages 235-242).

The eggs were inoculated on day 7 of incubation and incubation was continued the next day. Seven to nine days after inoculation of the virus into the yolk sac, the heads

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of the chicken embryos were removed and processed in accordance with the method described in Example 1. This entailed the final concentration of the viruses being carried out by precipitation with polyethylene glycol. The vaccine
5 prepared in this manner was subjected to the quality control tests described under I through VII. This vaccine also proved to be fully active in humans.

Example 5 - Preparation Of Quail Embryo Vaccine.

In a manner analogous to that described in Example 1,
10 attenuated rabies viruses were multiplied in quail eggs which had undergone initial incubation, and were harvested and processed to obtain the vaccine. The resulting vaccine proved to be fully active in animal experiments.

Example 6 - Preparation Of A Rabies Vaccine From Viruses

15 Multiplied In Mice.

Five-day old mice were inoculated intracerebrally with attenuated seed rabies viruses. After ten days, the mice which were still alive were sacrificed. The brains of the animals were removed and comminuted in a manner which
20 preserved cell and viral integrity. The cell suspension was processed to give a vaccine by the process of Example 1.

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Example 7 - Preparation Of A Rabies Vaccine From Viruses
Multiplied In Rats.

3-4 day old rats were inoculated intracerebrally with
attenuated seed rabies viruses. After 12 days, the rats
5 which were still surviving were sacrificed. The brains of the
animals were processed to give the vaccine in analogy to
Example 6.

Example 8 - Rabies Vaccine From Viruses Multiplied
In Rabbits.

10 Six day old rabbits were inoculated intracerebrally with
attenuated seed viruses. After 15 days the rabbits were
sacrificed. The brains of the animals were processed to give
a vaccine in analogy to Example 6.

Example 9 - Vaccine From Viruses Multiplied In Lambs.

15 8-10 day old sheep were inoculated intracerebrally with
attenuated seed rabies viruses. After 30 days, the lambs
were sacrificed. Their brains were removed and processed to
give a vaccine in analogy to Example 6.

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Having now fully described the invention, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit of the scope of the invention as set forth herein.

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WE CLAIM:

1. A process for obtaining inactivated rabies viruses, comprising:
 - (1) intracerebrally inoculating an experimental animal with whole live rabies viruses;
 - (2) allowing for said viruses to multiply;
 - (3) comminuting nerve tissue from the animal's brain to obtain a cell suspension, said comminution being conducted in the absence of a mixer to preserve the integrity of the viruses;
 - (4) separating live whole viruses from the cell suspension;
 - (5) delipidating the live whole viruses; and
 - (6) selectively concentrating the viruses; wherein steps (1) through (4) are conducted at least once and up to 3 times; said viruses being substantially myelin-free.
2. The process of claim 1 wherein the viruses are selectively concentrated by density gradient centrifugation or precipitation with a polyethylene glycol.
3. The process of claim 1 further comprising inactivating the viruses.

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4. The process of claim 1, wherein the viruses are separated from the cell suspension by washing and suspending thereof in a physiological buffer solution having a pH about 7 to 8.

5. The process of claim 1 wherein the viruses are delipidated by adding a water-immiscible liquid organic solvent.

6. The process of claim 1, wherein the solvent is a hydrocarbon selected from the group consisting of water-immiscible liquid hydrocarbons or halogenated hydrocarbons.

7. The process of claim 6 wherein the delipidating hydrocarbon is an halogenated hydrocarbon.

8. The process of claim 6 wherein the hydrocarbon is selected from the group consisting of a petroleum ether, fluorinated or chlorinated ethane and homologues thereof.

9. The process of claim 8 wherein the petroleum ether is heptanes.

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10. The process of claim 1 wherein the animal is selected from the group consisting of mice, rats, rabbits and sheep.

11. The process of claim 1 further comprising precipitation and concentrating the viruses.

12. The process of claim 3 wherein the viruses are inactivated by adding a virus-inactivating amount of β -propiolactone or tri-(n-butyl)phosphate.

13. The process of claim 1 further comprising placing said viruses in a sterile vial and freeze-drying thereof.

14. A rabies vaccine comprising inactivated rabies viruses which are substantially myelin-free in an amount effective to elicit an immunologizing response when administered to a subject.

15. The rabies vaccine of claim 14 having at least 10 antigen value units per mg of nitrogen.

16. The rabies vaccine of claim 14 in dosage unit form.

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17. A rabies vaccine comprising attenuated rabies viruses obtained by the process of claim 1, said vaccine being substantially myelin-free and said viruses being present in an amount effective to elicit an immunizing response when administered to a subject.

18. The rabies vaccine of claim 17 having at least 10 antigen value units per mg of nitrogen.

19. The rabies vaccine of claim 14 in dosage unit form.

20. A process for obtaining attenuated rabies viruses comprising

inoculating a poultry embryo egg with whole live rabies viruses;

allowing for said viruses to multiply;

comminuting the embryo from the poultry egg to obtain a cell suspension; said comminution being conducted in the absence of a mixer to preserve the integrity of the viruses;

separating live whole viruses from the cell suspension;

delipidating the live whole viruses; and

selectively concentrating the viruses; wherein steps (1) through (4) are performed at least once and up to 3 times; said viruses being substantially myelin-free.

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21. The process of claim 20 further comprising inoculating the viruses.

22. The process of claim 20 further comprising conducting the following steps at least once prior to inoculating the poultry embryo

intracerebrally inoculating an experimental animal with live whole rabies viruses;

allowing for the viruses to multiply; and

separating live whole viruses from brain tissue.

23. The process of claim 20, wherein the attenuated viruses are selectively concentrated by density gradient centrifugation or precipitation with a polyethylene glycol.

24. The process of claim 20, wherein the viruses are separated from the cell suspension by washing and suspending thereof in a physiological buffer solution pH about 7 to 8.

25. The process of claim 20 wherein the viruses are delipidated by adding a liquid water-immiscible organic solvent.

26. The process of claim 25, wherein the solvent is a hydrocarbon selected from the group consisting of liquid, volatile hydrocarbons or halogenated hydrocarbons.

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27. The process of claim 26 wherein the delipidating hydrocarbon is an halogenated hydrocarbon.

28. The process of claim 26 wherein the hydrocarbon is selected from the group consisting of a petroleum ether, fluorinated or chlorinated ethane and homologues thereof.

29. The process of claim 28 wherein the petroleum ether is heptane.

30. The process of claim 22 wherein the experimental animal is selected from the group consisting of mice, rats, rabbits and sheep.

31. The process of claim 21 wherein the viruses are inactivated by adding a virus-inactivating amount of α -propionolactone or tri-(n-butyl)phosphate.

32. The process of claim 20 further comprising precipitating and concentrating the viruses.

33. The process of claim 20 wherein the embryonic poultry eggs are selected from the group consisting of embryonic duck, chicken and quail eggs.

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34. The process of claim 20 further comprising placing said viruses in in sterile vial and freeze-drying thereof.

35. The process of claim 20 wherein the cell suspension containing the viruses is obtained by comminuting the beads of the embryos.

36. A rabies vaccine comprising inactivated rabies viruses obtained by the process of claim 20 said vaccine being substantially myelin-free and said viruses being present in an amount effective to elicit an immunizing response when administered to a subject.

37. The rabies vaccine of claim 36 containing at least 10 antigen value units per mg of nitrogen.

38. The rabies vaccine of claim 36 in dosage unit form.

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